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Year XVIII September & October 1944 Nos. 9 & 10

INTERNATIONAL INSTITUTE OF AGRICULTURE

# INTERNATIONAL BULLETIN OF PLANT PROTECTION

PUBLISHED MONTHLY

REPRINT FROM THE 'INTERNATIONAL REVIEW OF AGRICULTURE'

*CONTENTS*

Causes of error in the 'in vitro' control method of fungicides and description of the Palagi-Terzano 'nebulization bench', p. 65.

Third contribution to the locust bibliography (*continued*), p. 72.

IMPERIAL MYCOLOGICAL INSTITUTE 22 JUL 1946

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INTERNATIONAL BULLETIN
OF PLANT PROTECTION

1944 Nos. 9 & 10

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# CAUSES OF ERROR IN THE 'IN VITRO' CONTROL METHOD OF FUNGICIDES AND DESCRIPTION OF THE PALAGI-TERZANO 'NEBULIZATION BENCH' *

At the present stage of technical progress in 'in vitro' research on the fungicidal capacity of preparations, there are four basic methods :—

(1) With hand nebulizers, more or less improved, the technical details of which were improved after the preliminary experiments (REDDICK and WALLACE, 1916 ; WALLACE, BLODGETT and HESLER, 1911 ; MCCALLAN, 1930 ; MCCALLAN and WILCOXON, 1938 ; BORZINI, 1940 ; CANO, 1942) ;

(2) With immobile horizontal nebulizers (EVANS and MARTIN, 1935 ; HORSFALL, HEUBERGER, SHARVELLE and HAMILTON, 1940 ; MCCALLAN and WILCOXON, 1940) ;

(3) By means of deposit cylinders (MCCALLAN and WILCOXON, 1940) ;

(4) With the micro-pipette method (MONTGOMERY and MOORE, 1938).

Apart from this last method which starts from a different principle to that imagined by REDDICK and WALLACE and which has not become general in use, in all the other cases an attempt has been made to standardize experiment conditions, so that successive experiments can be effected under conditions as identical as possible.

In brief, the possible causes of error, already partly studied by MCCALLAN and WILCOXON, in laboratory toxicometric research may be the following :—

(1) General causes of error, such as cleanness of the slides, presence of toxic gases in the atmosphere of the laboratory, etc.

(2) Causes of error inherent in the fungus, from circumstances of conditions and time of development of the colonies, to manner of taking the conidia, the germinative variability of the different parent stock of the same species and the different clones of the same stock, etc.

(3) Causes of error inherent in nebulization (the only ones we will deal with in this study, referring solely to the technique adopted to eliminate them), and which vary in type and importance ; these regard :—

(*a*) nebulization pressure ;

(*b*) time of nebulization ;

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* Communication from Professors RAFFAELE CIFERRI and ELIO BALDACCI, Botanica Institute of the University and Italian Cryptogamic Laboratory, Pavia, Italy.

Complete details on the experiments described in this communication will be published in the Transactions of the Botanical Institute and the Cryptogamic Laboratory of Pavia.

(*c*) average diameter (minimum and maximum) of the drops which to a large extent should be attributed to the calibre of the nebulizer, etc. ;

(*d*) uniformity of aspersion of the cover-glasses which, in part, is in relation to the preceding factors, but also with factors (*b*) and (*c*), as well as with the nature of the nebulized surface (cover-glass) ; it is also connected with the 'wetability' of the nebulized fungicidal preparation ;

(*e*) uniformity of the aqueous suspension of the fungicidal product to be nebulized, etc.

(4) Causes of error inherent in contact between the sprinkled slides and suspensions of the conidia of the fungus regarding :—

(*a*) incomplete or unequal desiccation (or irregular solubilization) of the fungicidal product nebulized on the slide ;

(*b*) diverse density of the aqueous suspensions of the conidia ;

(*c*) volume of the suspension drops ;

(*d*) diameter of the suspension drops, etc.

(5) Causes of error inherent in the germination of the conidia and their calculation, which are not always easy to eliminate and which range from incubation temperature of the slides to the duration and other conditions of incubation, from the error (or personal coefficient of error) of the microscopist to methods of examining the drops under the microscope, etc.

(6) Causes of error inherent in correcting numerical data and their estimation, both in the case of correcting figures in relation to the controls (germination of conidia in suspension on non-nebulized slides and germination on slides nebulized with Bordeaux mixture or copper salts with an equivalent fungicidal power), for their numerical or graphic expression, and for the methods of comparing the fungicidal properties of the products under examination, etc.

The main effort of the scientists who preceded us tended to standardize the nebulization of the slides, which is one of the crucial factors of this technique, but, on the other hand, they do not seem to have given sufficient attention to other factors of equal importance such as the calibre of the suspension drops of the conidia (from the standpoint of both the volume of the drops and their diameter), and which we have endeavoured to take into account as well as the factors mentioned above.

Since the first experiments carried out in 1941, we found that the original method of REDDICK and WALLACE as it was employed in the beginning by MCCALLAN gave rise to appreciable differences in the results of the experiments effected parallelly. The technical improvement made by BORZINI only remedied this disadvantage very slightly.

The fundamental causes of error were at once identified with irregularity in aspersion of the slides, irregularity depending in its turn on :

(*a*) want of uniformity in the calibre of the nebulizers which, in harmony with the work done by MCCALLAN, was eliminated by using the same type of nebulizer (an American De Vilbiss) despite the difficulty in obtaining same, and subsequently with the construction of calibrated tubes ;

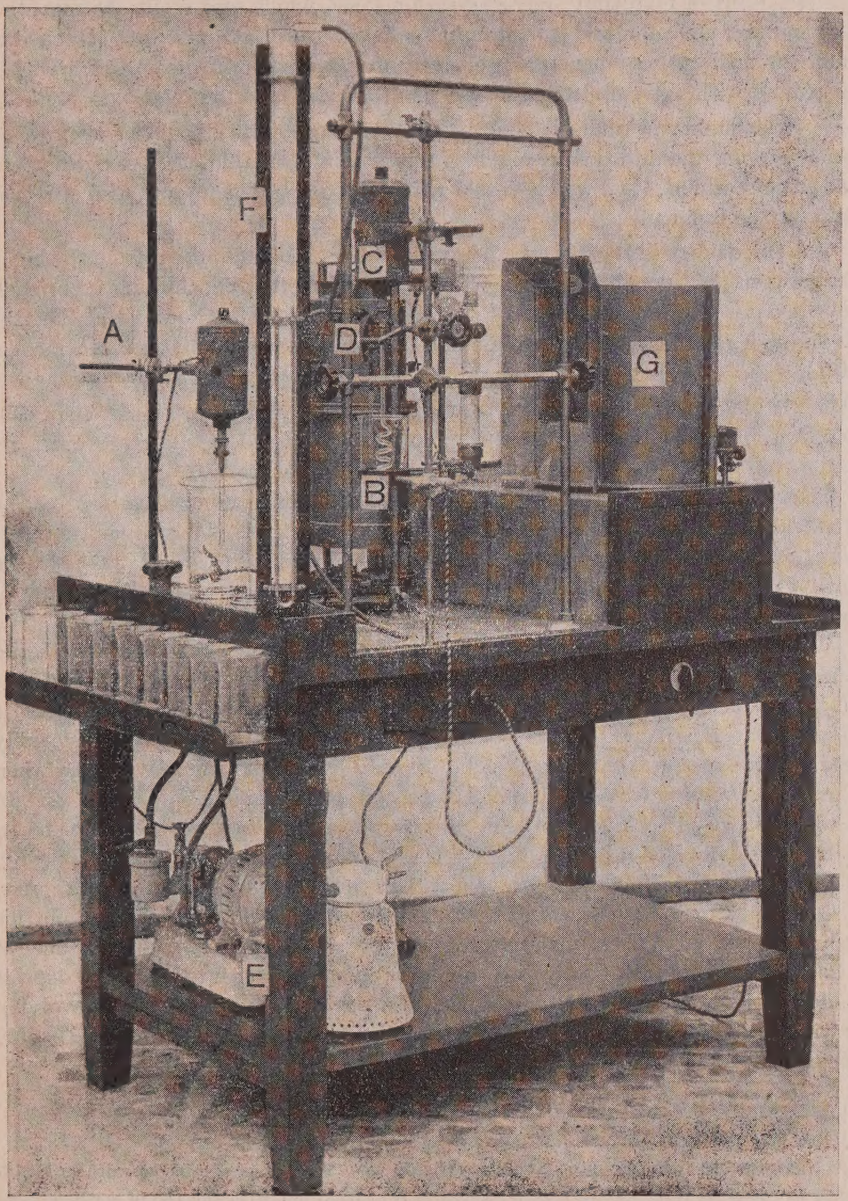


FIG. 1. — View of the 'bench for testing fungicides', PALAGI-TERZANO model.

(*b*) want of uniformity in nebulization force, which was easily be remedied by connecting the nebulizer to a receptacle having respiration action, attached to a mercury manometre (open tube), and, by maintaining uniform pressure with a rubber bulb with valve and then by means of a small air compressor ;

(*c*) inequality in time of nebulization of the slide, which could be avoided by chronometering the duration of nebulization and by ascertaining, through experiments, the optimum time which varied with factors (*a*) and (*b*) and also with the nature of the fungicide ;

(*d*) inequality in the distance between tube and slide (different from the equal level between the two) which can be eliminated by ascertaining through experimentation, what is the best distance for the drops of the fungicidal product to fall on the slide through gravity, although this distance varies at the same time as conditions (*a*) and (*b*).

After having modified these experiment conditions, and maintaining them constant from one experiment to another, appreciable differences, although much reduced, still occurred in the experiments carried out parallelly *.

These may have been due to some irregularities in conditions (*a*), (*b*) or (*c*), or to some defect in the tubes, or even to accidental causes such as air currents acting on the spray, coalescence of the drops before deposition, variation in diameter of the drops and consequently variation in rate of fall (and time of deposition, etc.).

Without seeking to ascertain the principal cause, we thought to range a series of slides on a revolving disc turning at reduced speed, using a small electric motor with adjustable speed for gramophone and the record holder covered with a circular glass plate of equal diameter. The plate was covered with a stable zinc box entirely closed save in one section, so as to nebulize only one of the slides at a time when the record on turning exposed the slide to the spray through the said opening. In this way, not only the timing of the nebulization was facilitated (reduced to calculation of the number of turns of the disc which could be stopped instantaneously), but also aspersion was more uniform since the spray descended by force of gravity.

Consequently, before knowing the latest investigations of McCALLAN and his collaborators on methods of nebulization, we were able to obtain the advantages of the horizontal nebulizer — easy and simple to operate — with those of the deposit cylinders — more accurate but more difficult to control — by means of the revolving disc which, so far as we know, has not been applied by other scientists in experiments on fungicides.

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* Not knowing at the time the latest investigations of McCALLAN and his co-workers (1940-41), experiments on the regularity of aspersion were carried out with the use of a copper sulphate solution of standard strength, subsequently thoroughly washing the slide with water, measuring it volumetrically after having operated on several slides simultaneously (from 30 to 50) ; then, according to the American investigator, by using a solution or aniline blue and measuring colorimetrically, after washing, the colour deposited on some slides.

Later Dr. E. PALAGI in collaboration with the firm of TERZANO & Co. of Milan * studied all the details of the construction of the apparatus (patented by this firm) making further improvements and increasing possible applications.

Besides the experiments which will be described, the Terzano-Palagi bench is fitted with attachments which enable experiments to be carried out on slides sprayed with the fungicide, such as experiments on action and resistance to cold or hot air currents, on the action of ultra-violet rays, etc., which we wil describe on another occasion.

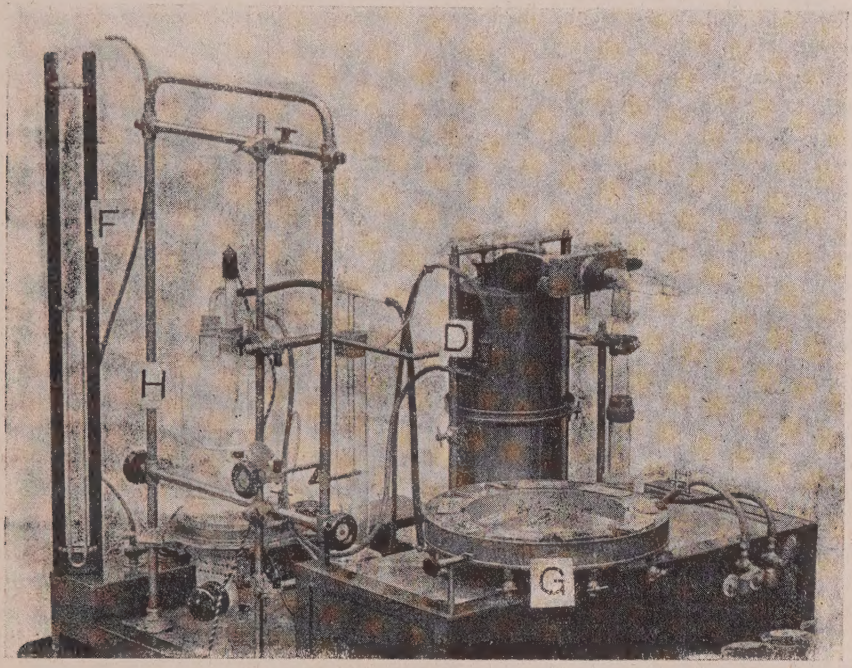


FIG. 2. — Close-up of the bench equipped for the nebulization of fungicides, showing the disc without the protective covering.

## I. — Experiments on the toxicity of liquid products.

Figure I shows the bench fitted with equipment for testing liquid preparations.

*A* represents the device serving for the preparation of the mixtures ; it consists of a stirrer by which the product is thoroughly mixed in the water before use. The solution obtained is then transferred into another glass *B* and always kept in move-

* We have to thank Dr. PALAGI and the TERZANO firm for permission to reproduce photographs of the apparatus and to publish description of same.

ment by stirrer *C*. Spraying is effected by means of two tubes arranged at right angles *D*, fed by the suction-compression pump arrangement *E*, while the column of the pressure gauge *F* indicates the pressure employed for spraying and which should be maintained constant. The product is finally sprayed on the slides by means of the device *G*.

The distance of the jet to the slides, the height of the jet in relation to the level of the slides, and the slant of the two tubes are regulated and kept constant by tubes with adjustment pins well to the fore of the bench.

Device *G* consists of a revolving disc with adjustable speed enclosed in a metal cylinder having a screened opening intended to protect the jet from outside influences such as air currents, etc. and at the bottom an inclined metal plate. The disc turns on its own axis and thus allows spraying of the fixed slides one after the other in a continuous manner (Fig. 2).

The special slides utilized for this apparatus have the shape and size of the usual microscopic slides, except for some slight differences which we will describe* ; ordinary slides can also be used.

In these slides four small circles were made so as to isolate a circular zone in the inner part of the circle. It is in this zone that the drop of water containing in suspension the spores of the fungus is deposited. The groove prevents the drop spreading during transport to and stay of the slides in the moist chamber and in the thermostats ; it also keeps the diameter of the drop constant.

The slides thus prepared are placed, as all experimenters do, in moist chambers formed by inverted Petri dishes. Since it is desirable for each drop to contain a constant number of conidia, a calibrated dropper was invented and adopted which made it possible to keep the suspension of the spores moving while depositing the drops, thanks to the aeration of the solution itself.

The slides, sprayed in the way already described for the experiment on toxicity, may be subjected to rain which resembles, under constant and analyzable conditions, the rain which falls in the country on the leaves. The quantity of the mixture and its residual fungicidal action which persist after this washing constitute two of the principal collateral characteristics of the greatest importance for a fungicidal product.

In order to approach as far as possible the conditions of adhesiveness of the mixture or wash on the cu icle of the vine leaves, the slides may first be covered with a layer of gum lac or nitrocellulose, so as to form ontheir surface a film or artificial membrane which resembles, in many characteristics, those of the leaf cuticle.

For the artificial rain use is made of the compressor-pump arrangement connected with a three-necked flask containing the water. The exact temperature of the water can be ascertained, while the air issuing from the tube communicating with

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* These slides and micropipettes were invented by Dr. PALAGI and manufactured by the TERZANO firm.

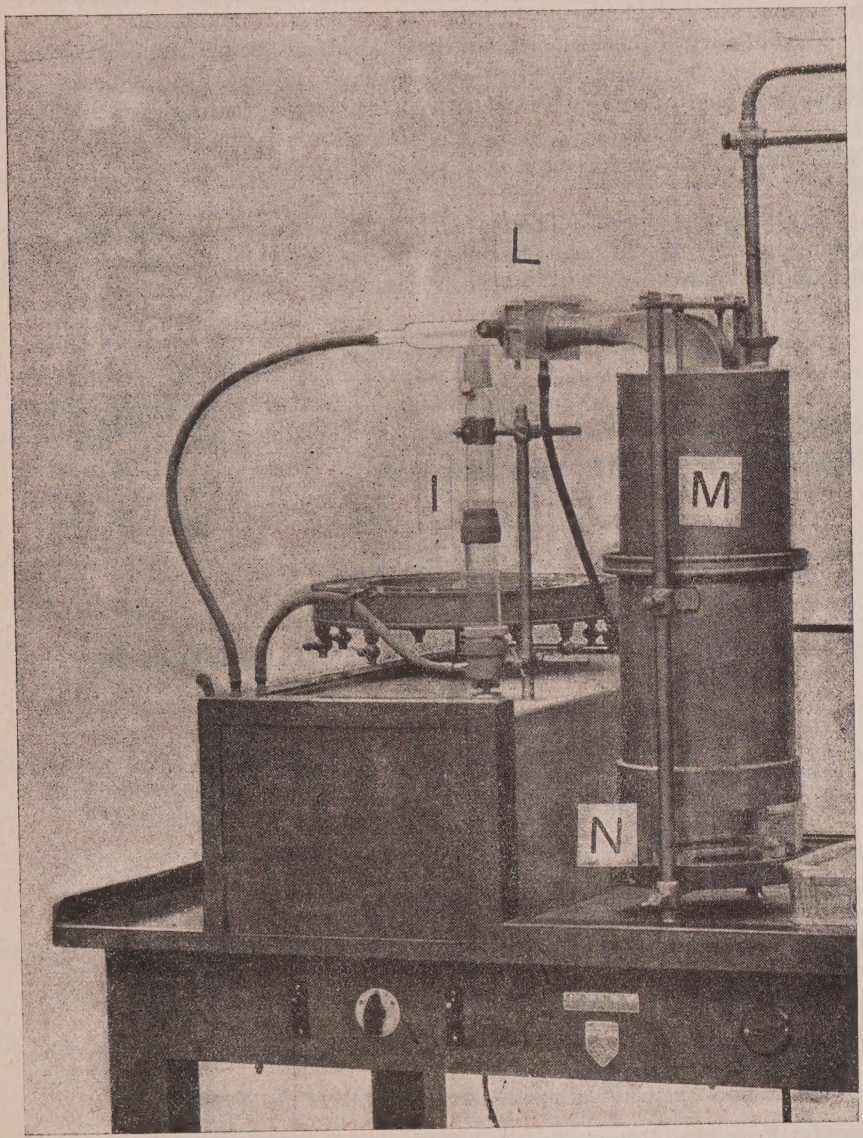


FIG. 3. — Detailed view of the bench fitted for testing dusts.

the compressor makes the water slightly carbonic. Carbon dioxide saturation can be obtained using a cylinder of this gas.

The intensity of this artificial rain is regulated and maintained constant in all the experiments by means of the pressure gauge *F* connected to the flask. The device which holds the slides is used without casing *G*, so that no obstacles are encountered and the rain spreads uniformly over the surface and the slides. The height and slant of the jet are usually regulated by tubes fitted with adjustment pins.

An attachment for controlling the height of the water falling on the slides within a given time consists of a small glass plate the size of the slides, in which are inserted four small graduated cylinders which indicate the height of the water.

II. — Experiments on toxicity of dusts.

The bench also has an arrangement for the treatment of slides with dusts these will be discussed on another occasion, a more improved and satisfactory technique still being under study (Fig. 3).

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* Drawn up by GIULIO TRINCHIERI, International Institute of Agriculture.

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(*To be continued*).

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Printed by F. FAILLI, Printing-Office of the International Institute of Agriculture
ROME, Villa Umberto I, November 30, 1944.

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